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Heterologous interactions between NS1 proteins from different influenza A virus subtypes/strains

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Non-structural protein 1 (NS1) of the influenza virus plays a crucial role in modulating the host immune response and facilitating virus replication. The formation of a homodimer or an oligomer is necessary for NS1 to exert its function efficiently. In the present study, the NS1 protein from the A/Shantou/602/06(H3N2) virus (herein abbreviated as NS32) was found to interact with NS1 from A/Shantou/169/06(H1N1), A/Chicken/Guangdong/1/05(H5N1) and A/Quail/Hong Kong/G1/97(H9N2) (abbreviated as NS11, NS51 and NS92, respectively) viruses, although NS32 shares 17.4%–20.9% sequence diversity with NS11, NS51 and NS92. This indicates that the heterologous interactions between NS1 proteins from different influenza A virus subtypes/strains may be a common event during co-infection.

influenza A virus, NS1 protein, heterologous interaction

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The influenza A virus is a common pathogen that causes an acute respiratory infection. According to the antigenicity of hemagglutinin and neuraminidase, the influenza A virus can be divided into numerous subtypes. Currently, at least 16 HA-subtypes and 9 NA-subtypes have been reported [1]. All known subtypes can be found in wild birds, whereas a few subtypes are present in some mammalian species, such as humans, pigs, horses and seals.

The RNA genome of the influenza A virus is composed of eight segments that encode 11 proteins. The segmented genome is favorable for the evolution of the influenza virus via a process called reassortment. Reassortment takes place in permissive host cells when they are co-infected by at least two different influenza viruses. In general, the details for co-infection and gene reassortment are far from clear, since they are implicated in complex protein-protein and protein-RNA interactions between different viruses or between virus and host cells.

As a non-structural protein of the influenza virus, non-structural protein 1 (NS1) is highly expressed in infected cells. NS1 mainly serves as an antagonist of the host immune response or an inhibitor that blocks the processing and nuclear export of host mRNA [2]. Two domains, an N-terminal RNA-binding domain and a C-terminal effector domain, have been identified in the NS1 protein [2]. Both the RNA-binding domain [3–6] and the effector domain [7,8] have been shown to form dimers individually. More importantly, dimerization or oligomerization is crucial for

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the NS1 protein to interact with RNA [9] and counteract an IFN response [10], suggesting the homologous interactions between NS1 proteins from the same influenza virus play an important role in the infectious process.

In the present study, we provide evidence for the heterologous interactions between NS1 proteins from different influenza A virus subtypes/strains, which have not been reported previously. The results contribute to a better understanding of the complicated biological processes present in mixed influenza viruses infections.

1 Materials and methods

1.1 Cell lines, viruses and reagents

The human lung carcinoma cell line (A549) and human cervix epithelial cells (Hela) were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) containing antibiotics and 10% fetal calf serum at 37°C in 5% CO_2.

Influenza A virus strains A/Shantou/169/06(H1N1), A/Shantou/602/06(H3N2) and A/Chicken/ Guangdong/1/05(H5N1) and viral RNA from the A/Quail/Hong Kong/G1/97(H9N2) strain were used in this study. The viruses were abbreviated as ST169, ST602, GD05 and Qa97, respectively.

Trizol reagent was purchased from Invitrogen (Carlsbad, CA, USA), whereas AMV reverse transcriptase, PrimeSTAR HS DNA polymerase, restriction endonucleases and T4 DNA ligase were from TaKaRa (Dalian, China).

Mouse anti-Flag antibody and peroxidase-conjugated goat anti-mouse antibody were from Sigma (St. Louis, MO, USA). Rabbit anti-HA antibody was from Cell Signaling (Danvers, MA, USA), whereas mouse anti-NS1 antibody was from Santa Cruz (Santa Cruz, CA, USA). Mouse anti-HA antibody was from Tiangen (Beijing, China), and peroxidase-conjugated goat anti-rabbit antibody and Cy3-conjugated goat anti-mouse antibody were from Beyotime Biotechnology (Jiangsu, China).

The yeast MATCHMAKER GAL4 two-hybrid system 3 and X-α-gal were from Clontech (Palo Alto, CA, USA); the MagneGST pull down system, TNT T7 Quick Coupled Transcription/Translation Systems and Transcend Chemiluminescent Non-Radioactive Translation Detection Systems were from Promega (Madison, WI, USA); Protein A/G magnetic beads were from New England Biolabs (NEB, Ipswich, MA, USA); protein G-HRP was from Genescript (Piscataway, NJ, USA); and West Dura enhanced chemiluminescence reagents were from Pierce (Rockford, IL, USA).

1.2 Plasmids construction

NS1-encoding sequences from different Influenza A viruses (A/Shantou/169/06(H1N1), GenBank: HQ849876; A/ Shantou/602/06(H3N2), GenBank: HQ849877; A/chicken/Guangdong/1/05(H5N1), GenBank: EU874904; A/Quail/Hong Kong/G1/97(H9N2), GenBank: AF156477) were amplified by reverse transcription PCR using viral RNAs and the following primer sets: NS11-S1: 5′-AATGGATCCATGGATTCCCACACTGT-3′ and NS11-A1:5′-TCGGGATCCTCAAACTTCT GACCTAAT-3′ for A/Shantou/169/06(H1N1); NS32-S1: 5′-TATGGATCCATGGATTCCAACACTGTG-3′ and NS32-A1: 5′-TACGGATCCTCAAACTTTTGACCTAGC-3′ for A/Shantou/602/06(H3N2); NS51-S1: 5′-TATGGATCCATGGATTCCAACACTGTG-3′ and NS51-A1: 5′-GACGGATCCTCAAACTTTTGACT CAATTG-3′ for A/Chicken/Guangdong/1/05(H5N1); and NS92-S1: 5′-TATGGATCCATGGATTCCAACACTGTG-3′ and NS92-A1: 5′-AGTGGATCCTCAAACTTCTGGCTCAAT-3′ for A/Quail/Hong Kong/G1/97(H9N2). PCR products were digested with *Bam*H I and inserted into the PNF vector (a modified pcDNA3 vector with an N-terminal Flag tag), pGEX-6p-1 vector, pcDNA3 vector, or pEGFP- C1 vector, to give the recombinant plasmids Flag- NS11, Flag-NS32, Flag-NS51, Flag-NS92, pGEX-NS11, pGEX-NS32, pGEX-NS51, pGEX-NS92, pcDNA3-NS32 and pEGFP-NS32. NS11, NS32, NS51 and NS92 herein are the abbreviations of NS1 proteins from H1N1, H3N2, H5N1 and H9N2 viruses, respectively.

To construct NS1-expressing plasmids used for the yeast two-hybrid assay, the following primers were designed: NS11-S2: 5′-ACTGAATTCATGGATTCCCACACTGTG-3′; NS32- S2: 5′-CGTGAATTCATG GATTCCAACACTGTG-3′; NS51-S2: 5′-TATGGATCCTTATGGATTCCAACACTGTG-3′; and NS92-S2: 5′-CGTGAATTCATGGATTCCAACACTGTG-3′. The reverse transcription PCR reactions were performed using primer sets NS11-S2 and NS11-A1 for A/Shantou/169/06(H1N1), NS32-S2 and NS32-A1 for A/Shantou/602/06(H3N2), NS51-S2 and NS51-A1 for A/Chicken/Guangdong/1/05(H5N1), and NS92-S2 and NS92-A1 for A/Quail/Hong Kong/G1/97 (H9N2). PCR products were digested with appropriate enzymes and cloned into either the pGADT7 or pGBKT7 vector to yield plasmids pGAD-NS11, pGAD-NS32, pGAD-NS51, pGAD-NS92 and pGBK-NS32. To generate plasmid pcDNA-HA-NS32, the full-length NS1-coding sequence of A/Shantou/602/06(H3N2) was amplified using primer sets NS32-S1 and NS32-A2 (5′-TACGAATTCTCAAACTTTTGACCTAGC-3′) by reverse transcription PCR and fused into pcDNA-HA (a modified pcDNA3 vector with an N-terminal HA tag) via *Bam*H I and *Eco*R I sites.

All recombinant plasmids were verified by sequencing.

1.3 Yeast two-hybrid assay

The yeast two-hybrid assay was performed using the MATCHMAKER GAL4 two-hybrid system 3 according to the manufacturer's instruction. Briefly, AH109 yeast was transformed with plasmids pGAD-NS11, pGAD-NS32, pGAD-NS51 and pGAD-NS92 along with pGBK-NS32 and

plated onto the synthetic dropout media SD/-Leu/-Trp (DDO) and SD/-Ade/-His/-Leu/-Trp (QDO). AH109 yeasts transformed with plasmids pGBKT7-lam plus pGADT7-T or pGBKT7-P53 plus pGADT7-T were used as negative and positive controls, respectively. The plates were incubated at 30°C for ~4 d. Fresh AH109 colonies growing on DDO plates were picked and transferred to QDO plates containing X-α-gal (QDO/X-α-gal) followed by incubation at 30°C for 2 or 3 d. The growth and color of colonies were observed daily.

1.4 GST pull-down analysis

Escherichia coli BL21 was transformed with pGEX-6p-1 or recombinant plasmids encoding NS1 proteins and grown to mid-log phase. IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 0.1 mmol L^{-1}. The cultures were incubated with shaking (200 cycles min^{-1}) at 25°C for 4 h. After 2 cycles of freezing and thawing, bacterial pellets were lysed with the MagneGST lysis reagent containing lyticase, DNase and protease inhibitors for 40 min, followed by centrifugation at 14000×*g* for 10 min. The supernatants were mixed with pre-equilibrated MagneGST beads at 4°C for 30 min. The GST-binding or GST-NS1-binding beads were washed with binding/wash buffer four times and the bound GST or GST-NS1 was separated by a 12% SDS-PAGE gel and detected by Coomassie blue staining.

In vitro transcription/translation of NS32 was conducted using the pcDNA3-NS32 plasmid and TNT T7 Quick Coupled Transcription/Translation Systems, according to the protocol of the manufacturer. The expression of the NS32 protein was examined by Western blot analysis using an antibody against NS1.

GST or GST-NS1 beads were subsequently incubated with the NS32 protein at room temperature for 1.5 h. After six extensive washing steps with binding/wash buffer, the pull-downed proteins were resolved on a 12% SDS-PAGE gel and blotted with anti-NS1 antibody.

1.5 Co-immunoprecipitation (Co-IP) test

Hela cells were transfected with Flag-tagged NS1-expressing plasmids and a HA-tagged NS32-expressing plasmid, individually or in combination. At 36-h post transfection, cells were lysed in cold NP-40 lysis buffer on ice for 20 min and centrifuged at 14000×*g* for 10 min. Supernatants were pretreated by protein A/G magnetic beads for 1 h. The samples were then incubated with rabbit anti-HA antibody (1:300) at 4°C for 2 h. Normal rabbit IgG was applied to serve as a control. Protein-antibody mixtures were further incubated overnight with protein A/G magnetic beads at 4°C with gentle rotation. After extensive washes with the NP-40 lysis buffer, the precipitated proteins were run on a 12% SDS-PAGE gel and probed with the mouse anti-Flag antibody (1:800) or mouse anti-HA antibody (1:1000) as well as Protein G-HRP (1:2500). In addition, cell lysates were directly subjected to SDS-PAGE and Western blotting with the indicated antibodies.

1.6 Immunofluorescence microscopy

Confluent A549 cells were transfected with the pEGFP-NS32 plasmid along with different NS1-expressing plasmids or the empty vector PNF. At 24-h post transfection, cells were washed with PBS, fixed with 4% paraformaldehyde at room temperature for 10 min, permeabilized with 0.2% Triton X-100 for 10 min, and blocked in PBS containing 3% BSA for 30 min. Subsequently, cells were stained with mouse anti-Flag antibody (1:500) diluted in 3% BSA-PBS for 2 h. After washing with PBS three times, the cells were incubated with Cy3-conjugated goat anti-mouse antibody (1:300) for 1 h, followed by 5 min of nuclei-staining with Hoechst33258 (1:1000). Finally, digitized images were captured by a Zeiss (Axio Imager Z1) fluorescent microscope.

2 Results

2.1 NS1 proteins from different subtypes vary in their amino acid sequences

Sequence alignment results showed that NS1 proteins from four influenza virus subtypes/strains differ in their sequence length and amino acids composition. As shown in Figure 1, NS1 proteins from influenza viruses A/Shantou/169/06(H1N1), A/Shantou/602/06(H3N2) and A/Quail/Hong Kong/G1/97(H9N2) (abbreviated as NS11, NS32 and NS92) are 230 residues in length, whereas NS1 from the A/chicken/Guangdong/1/05(H5N1) virus (NS51) is only 225 residues in length because of a unique five residue deletion between position 80 and 84, which is a typical feature of NS1 proteins from H5N1 viruses isolated since 2000 [11,12]. The variability in protein sequence between NS32 and NS1 from three other viruses (NS11, NS51 and NS92) is 17.4%–20.9%. In agreement with other reports [13,14] , the variation sites mainly distribute in the C-terminal effector domain (74–230 aa), in particular, at the very end of the C-terminus.

2.2 Examination of the interactions between NS32 and other NS1 proteins using a yeast two-hybrid assay

To test the possible interactions between NS1 proteins from different influenza A virus subtypes/strains, a yeast two-hybrid assay was employed. Five recombinant plasmids (pGBK-NS32, pGAD-NS11, pGAD-NS32, pGAD-NS51, and pGAD-NS92) were constructed, in which NS1 encoding sequences from different influenza A viruses were fused to either the DNA-binding domain or the activation domain

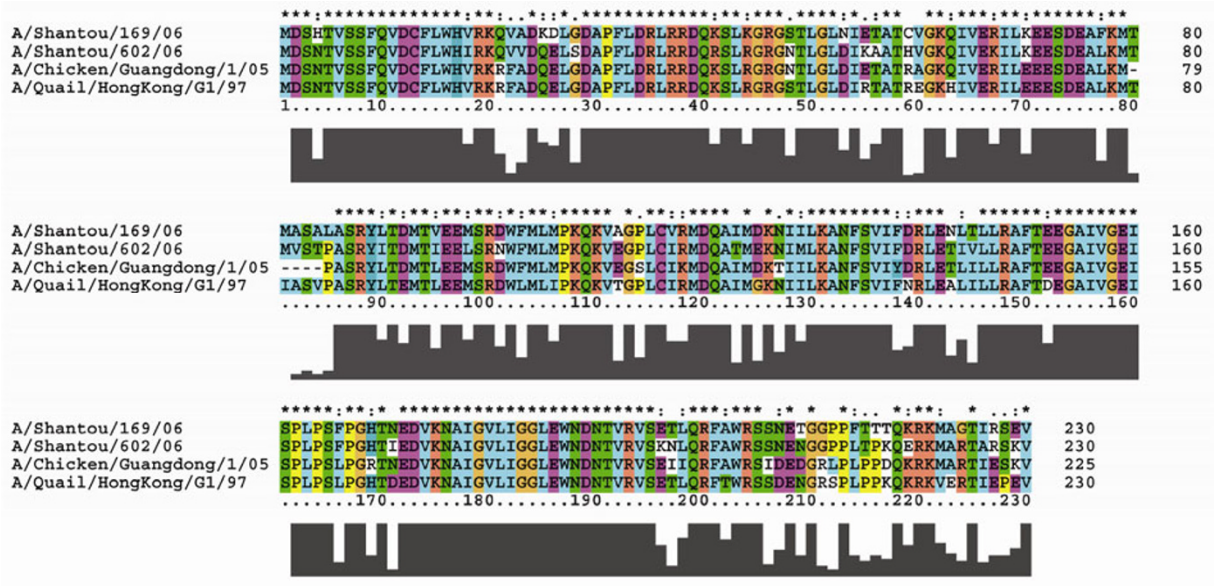

Figure 1 Schematic diagram depicting the sequence variation among different NS1 proteins. Asterisks above the sequences mark the conserved amino acids. Please note that there are five residues missing between positions 80 and 84 of NS1 from the A/chicken/Guangdong/1/05(H5N1) virus.

of Gal4. These plasmids can be used in the subsequent two-hybrid assay, because they do not have auto-activating ability when transformed into AH109 yeasts individually (data not shown).

Our results showed that, similar to the positive control (pGBKT7-p53 plus pGADT7-T), AH109 yeasts harboring each pGADT7-based NS1-expressing plasmid along with the pGBK-NS32 plasmid exhibited vigorous growth on the QDO plates (Figure 2A) and formed characteristic blue colonies on the QDO/X-α-gal plates (Figure 2B), indicating the activation of the reporter gene and the secretion of α-galactosidase in these yeast cells. In contrast, AH109 yeast cells in the negative control group (pGBKT7-Lam plus pGADT7-T) did not grow on the QDO plates (Figure 2A), nor did they produce and secrete α-galactosidase on the QDO/X-α-gal plate (as shown in Figure 2B, the yeasts turned brown and did not survive). These results suggest the interactions of NS32 with NS11, NS51 or NS92 in yeast cells.

2.3 *In vitro* interactions between NS32 and other NS1 proteins

We next performed a GST pull-down analysis to detect the *in vitro* interactions between NS32 and other NS1 proteins. Four different NS1 proteins with an N-terminal GST tag were expressed and immobilized on MagneGST beads (Figure 3A). The NS32 protein was translated using a cell-free system and identified by Western blot (Figure 3B). When NS32 was mixed with GST- or GST-NS1-binding beads, it can be efficiently pulled down by immobilized GST-NS11, GST-NS32, GST-NS51, or GST-NS92 proteins, but cannot be precipitated with GST, as revealed in Figure 3C.

2.4 *In vivo* interactions between NS32 and other NS1 proteins

To further examine whether the NS32 protein can interact with NS1 proteins from other influenza A virus subtypes/strains in mammalian cells, co-IP tests were applied. Flag-NS11, Flag-NS51, or Flag-NS92 could easily be detected in protein complexes precipitated by the HA-antibody (lane 5 in the upper panels of Figure 4A–C) in Hela cells co-transfected with plasmids encoding HA-NS32 and Flag-tagged NS1 proteins. Nevertheless, none of the NS1 proteins was precipitated when normal rabbit IgG was used (lane 4 in the upper panels of Figure 4A–C). Moreover, no NS1 protein bands were detected in cells transfected with either the HA-NS32 or Flag-NS1 plasmid alone (lanes 1 and 2 in the upper panels of Figure 4A–C). The above phenomena suggest the specific interplay between NS32 and other NS1 proteins *in vivo*.

2.5 Co-localization of NS32 and other NS1 proteins in A549 cells

NS1 is known to be a nuclear protein. When different Flag-tagged NS1 proteins were exogenously expressed in A549 cells, they predominantly exhibited a diffused distribution with some discrete dots in the nuclei (column 1 in Figure 5), as was also observed for the GFP-NS32 fusion construct (column 2 in Figure 5). Apparently, overlapping localization of most GFP-NS32 protein and Flag-NS11, Flag-NS51, or Flag-NS92 were observed in co-transfected cells (yellow arrows in Figure 5). Furthermore, although GFP-NS32 displayed an almost entirely identical localization pattern with the Flag-NS32 protein, a few GFP-NS32

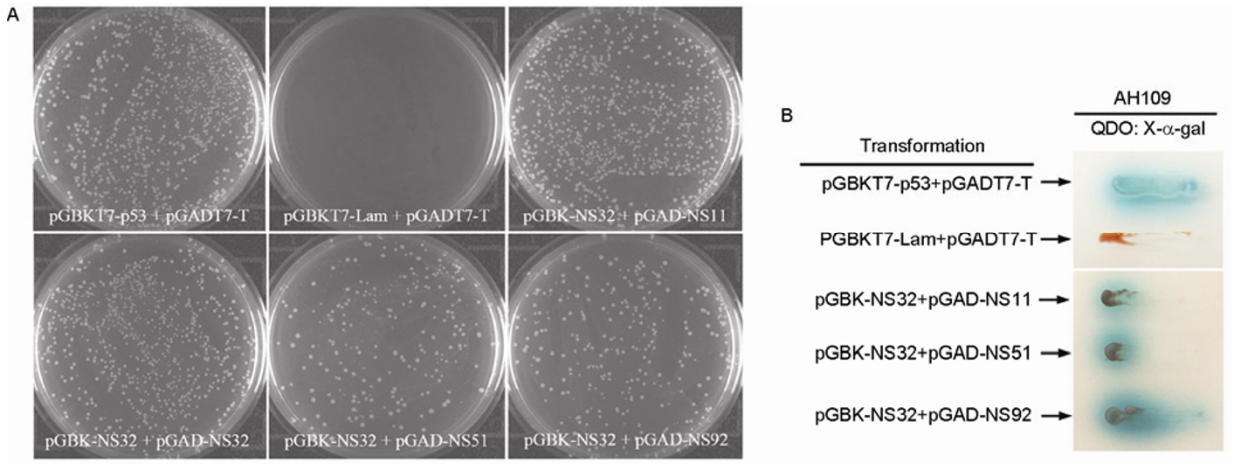

Figure 2 Yeast two-hybrid analysis of the interactions between NS32 and other NS1 proteins. A, AH109 yeasts were transformed with pGBK-NS32 plasmids together with different pGADT7-based NS1-encoding plasmids and plated on SD/-Ade/-His/-Leu/-Trp (QDO) agar plates for 4 d of incubation at 30°C. B, AH109 yeasts transformed with the indicated plasmids were first plated onto SD/-Leu/-Trp (DDO) plates for 4 d of incubation at 30°C. Single AH109 colonies were then picked and streaked onto QDO/X-α-gal agar plates and incubated at 30°C for 3 d. The representative colonies were presented. pGBKT7-p53+pGADT7-T and pGBKT7-Lam+pGADT7-T represented positive and negative controls, respectively.

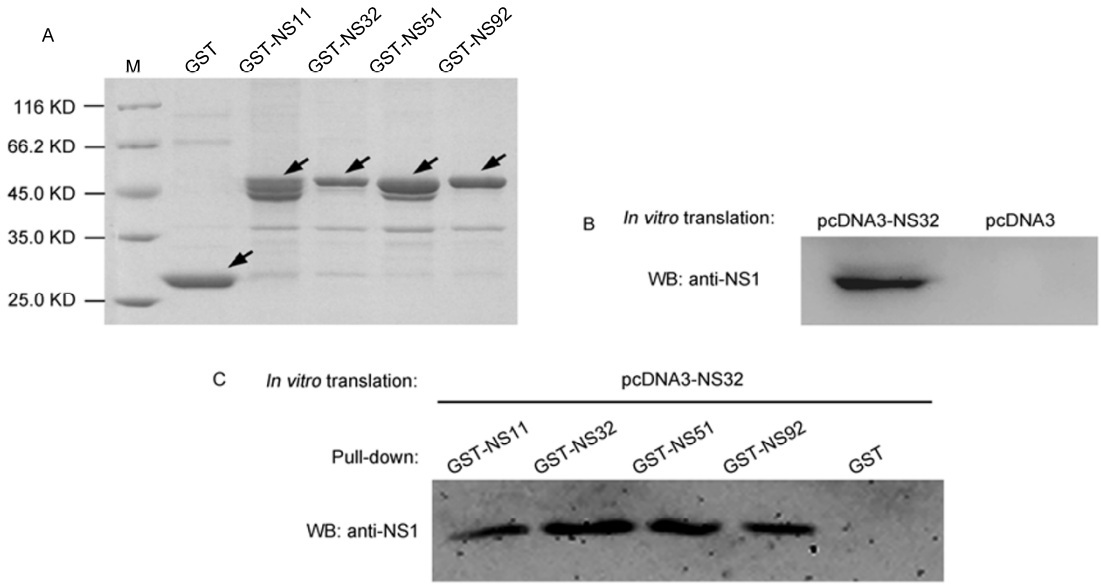

Figure 3 *In vitro* binding of NS32 with other NS1 proteins. A, Expression of GST or GST-NS1 proteins in *E. coli* BL21 and their immobilization on magnetic beads. The immobilized GST or GST-NS1 proteins were resolved on SDS-PAGE and examined by direct staining with Coomassie brilliant blue; M, molecular weight standards. B, *In vitro* translation of the NS32 protein using the pcDNA3-NS32 plasmid and TNT T7 Quick Coupled Transcription/Translation Systems. The translated NS32 was verified by Western blot analysis using anti-NS1 as the primary antibody. C, Pull-down assay. NS32 was incubated with GST, GST-NS11, GST-NS32, GST-NS51, or GST-NS92 immobilized onto magnetic beads. After washing, protein complexes were dissociated from the beads and subjected to Western blotting with the anti-NS1 antibody.

molecules had located to nuclear sites without the distribution of Flag-NS11, Flag-NS51, or Flag-NS92 (white arrows in Figure 5), implying that NS32 cannot efficiently interact with these NS1 proteins in these sites within nuclei.

3 Discussion

Co-circulation of multiple influenza virus subtypes/strains in the same epidemic season and the subsequent mixed infection of an individual by two or more influenza viruses provides an opportunity for genetic reassortment. It has been established that reassortment is an important evolutionary mechanism for human influenza viruses that has led to the emergence of global pandemics of human influenza viruses in 1957 (H2N2) [15–17], 1968 (H3N2) [15,16] and 2009(H1N1) [18].

Despite annual co-circulation of different influenza virus

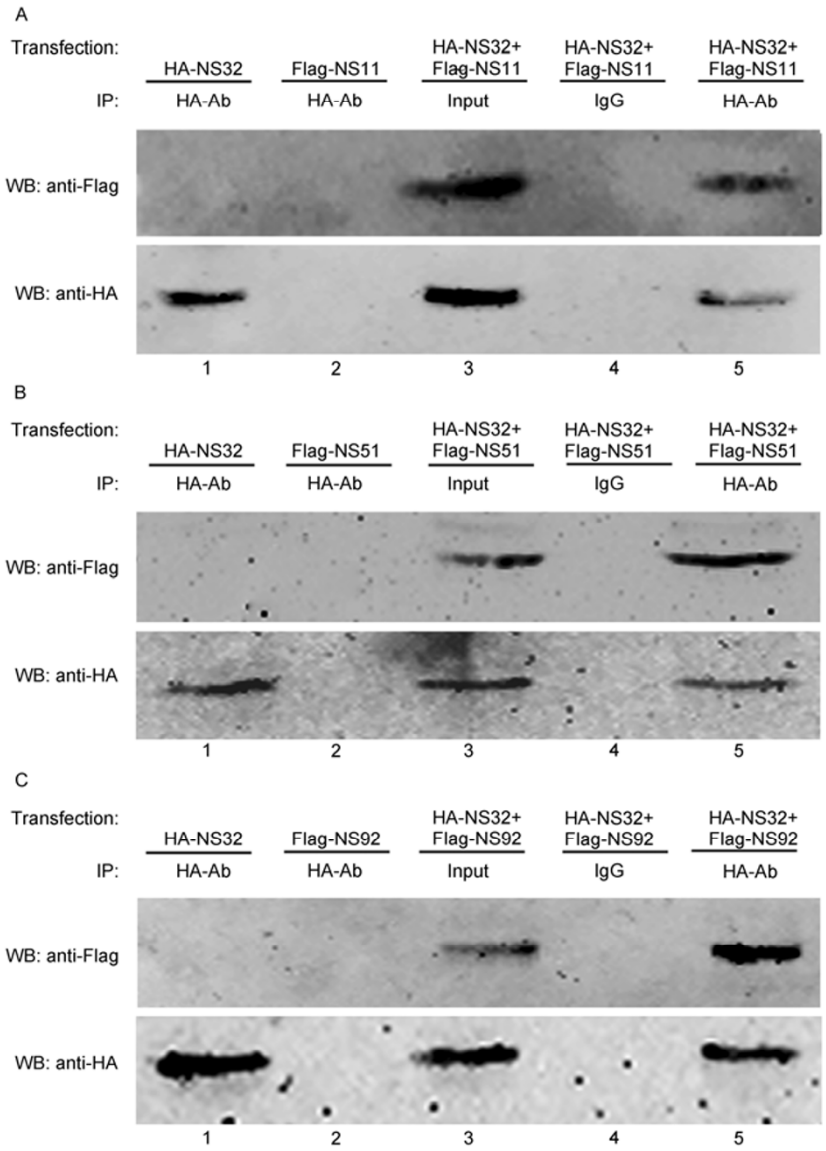

Figure 4 *In vivo* association of NS32 with other NS1 proteins. HA-NS32-expressing plasmid and Flag-tagged NS1-expressing plasmids were transfected into Hela cells individually or together. After 36 h of transfection, soluble cellular lysates were immunoprecipitated with the rabbit anti-HA antibody or the normal rabbit IgG. Precipitated proteins were resolved by 12% SDS-PAGE gels, followed by Western blotting analysis using the mouse anti-Flag antibody or the mouse anti-HA antibody.

subtypes/strains, co-infection of influenza viruses in humans is believed to be a rare event [19]. The co-infection rate determined in one study performed recently in New Zealand was 1.1% [20], whereas another study estimated this rate to be about 3% [21]. However, the actual rate may be under-estimated because current diagnostic methods (e.g., multiple PCR) cannot cover all possible infectious combinations of influenza viruses. In addition, compared to humans, the co-infection rate could be much higher among avian species. For example, Dugan and colleagues found that up to 26% of the isolates from wild-birds experienced mixed infection of different subtypes [22].

Mixed infection with the pandemic (H1N1) 2009 virus and the seasonal (H1N1 or H3N2) influenza viruses, even the high pathogenetic H5N1 avian influenza virus has created considerable concern, because of the possible generation of a virus that can acquire new properties of virulence, transmissibility and drug resistance. However, it is noteworthy that co-infection of an individual by influenza A viruses is not a random event; particular virus combinations may appear more often or less often than one can expect from the rate of single-virus infections [23]. The underlying mechanism responsible for this phenomenon remains largely unknown, but it is likely to be correlated with the reciprocal influence of different influenza virus strains, which means viral components from one virus may facilitate or restrict the infectious course of another virus in the same set of cells.

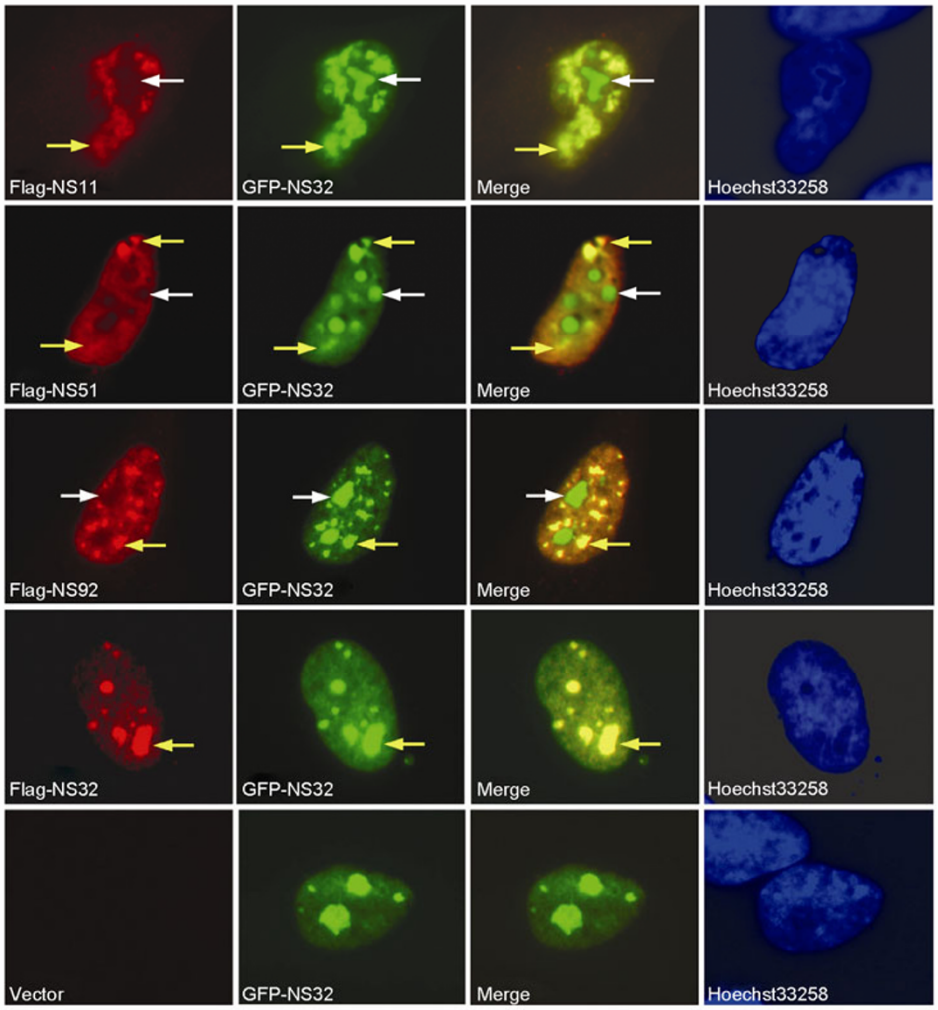

Figure 5 Immunofluorescence analysis of the localization relationship between NS32 and other NS1 proteins. The GFP-NS32-expressing plasmid was transfected into A549 cells in combination with different recombinant plasmids encoding Flag-NS1 (namely Flag-NS11, Flag-NS32, Flag-NS51, or Flag-NS92) or empty vector PNF (a modified pcDNA3 vector with an N-terminal Flag tag). At 24-h post transfection, cells were immunostained with the mouse anti-Flag antibody and the Cy3-labeled goat anti-mouse secondary antibody. Nuclei were counterstained with Hoechst 33258. Localization of the Flag-NS1 protein (red), GFP-NS32 (green), overlaid images of Flag-NS1 proteins and GFP-NS32 (yellow), and nuclei (blue) are shown. Yellow and white arrows indicate the same and different localization patterns, respectively, of the GFP-NS32 protein with Flag-NS11, Flag-NS51, or Flag-NS92.

In our study, a series of experiments (yeast two-hybrid assay, GST pull-down, co-immunoprecipitation, and immunofluorescence analysis) were performed to examine the interactions between NS1 proteins from different influenza A virus subtypes/strains. Here, NS1 proteins from four different influenza A virus subtypes (NS11, NS32, NS51 and NS92) were used and we selected NS1 from the A/Shantou/602/06(H3N2) virus as the major subject. This is because NS1 proteins from many H3N2 viruses can locate to the nucleoli of mammalian cells, which has not been observed for most NS1 proteins from other influenza virus subtypes [24–26]. In our previous study, we also noticed a distinct distribution pattern of NS32 in Hela cells with NS11, NS51 and NS92 [27]. The unique nucleolus localization of NS32 protein may modulate the synthesis of host rRNA and hence affect the pathogenicity of the influenza virus [26,28]. Furthermore, we found that the NS32 protein exhibited remarkable variation in the transcription-stimulating capability in yeast when compared to NS11, NS51 and NS92 [29]. In the present study, we further show that NS32 is able to efficiently interact with NS11, NS51 and NS92 both *in vitro* and *in vivo*, albeit harboring 17.4%–20.9% sequence diversity compared with the other three NS1 proteins (Figure 1). This is the first report regarding heterologous interactions between different NS1 proteins.

Results from a previous study indicated that NS1 functions as a homodimer or multimer *in vivo* [30]. A more recent study further described a unique tubular structure based on the homologous NS1 multimer [31]. This structure confers extreme flexibility to NS1 and enables NS1 to bind different lengths of dsRNA [31]. Apart from its RNA-binding activity, NS1 can also interact with a number of

cellular proteins to facilitate the replication of the influenza virus. The average length of NS1 is 230 residues. However, the variations of NS1 in amino acids sequence such as truncation, deletion, insertion or residue substitutions are popular among all subtypes of the influenza A virus [14]. The variations sometimes cause an alternation in the function of NS1. For example, F103S and M106I mutations in NS1 abrogated its ability to bind CPSF30 (cleavage and polyadenylation specificity factor 30 kD) [32]. Additionally, because of sequence variation, NS1 from different influenza viruses exerts differential inhibitory effects on RNAi [33]. Although NS32 used in this study shares about 82.6%, 79.6% and 79.1% identity with NS11, NS51 and NS92, respectively (Figure 1), these NS1 proteins differ in their transcription-activating ability in yeast cells [29] and PI3K-activating ability in mammalian cells [34]. Accordingly, we cannot exclude the possibility that the heterologous interactions between different NS1 proteins may affect the formation of highly ordered spatial structures and/or the biological function of the NS1 dimer or multimer. However, our attempt to explore the functional significance of the heterologous NS1 interactions was hampered by the fact that the biological effects induced by the simultaneous expression of two different NS1 proteins in one cell may result from the competitive binding of different NS1 proteins with the same host factor but not NS1-NS1 interactions.

In summary, heterologous interactions between different NS1 proteins during co-infection appear to be a common phenomenon, but its role on the severity and outcome of co-infection awaits clarification.

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